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## **Novel bone substitute material in alveolar bone healing following tooth extraction: an experimental study in sheep**

Liu, Jinyi ; Schmidlin, Patrick R ; Philipp, Alexander ; Hild, Nora ; Tawse-Smith, Andrew ; Duncan, Warwick

**Abstract:** **OBJECTIVES:** Electrospun cotton wool-like nanocomposite (ECWN) is a novel synthetic bone substitute that incorporates amorphous calcium phosphate nanoparticles into a biodegradable synthetic copolymer poly(lactide-co-glycolide). The objectives of this study were to develop a tooth extraction socket model in sheep for bone graft research and to compare ECWN and bovine-derived xenograft (BX) in this model. **MATERIAL AND METHODS:** Sixteen cross-bred female sheep were used. Bilateral mandibular premolars were extracted atraumatically. Second and third premolar sockets were filled (Latin-square allocation) with BX, ECWN or left unfilled. Resorbable collagen membranes were placed over BX and selected ECWN grafted sockets. Eight sheep per time period were sacrificed after 8 and 16 weeks. Resin-embedded undemineralised sections were analysed for descriptive histology and histomorphometric analyses. **RESULTS:** At 8 weeks, there were with no distinct differences in healing among the different sites. At 16 weeks, osseous healing followed a fine trabecular pattern in ECWN sites. Non-grafted sites showed thick trabeculae separated by large areas of fibrovascular connective tissue. In BX grafted sites, xenograft particles were surrounded by newly formed bone or fibrovascular connective tissue. There were no statistically significant differences in bone formation across the four groups. However, ECWN sites had significantly less residual graft material than BX sites at 16 weeks ( $P = 0.048$ ). **CONCLUSIONS:** This first description of a tooth extraction socket model in sheep supports the utility of this model for bone graft research. The results of this study suggested that the novel material ECWN did not impede bone ingrowth into sockets and showed evidence of material resorption.

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## Title Page

Novel bone substitute material in alveolar bone healing following tooth extraction: an experimental study in sheep

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### **Keywords**

animal, biomaterials, bone substitutes, extraction socket, wound healing

## Abstract

**Objectives:** Electrospun cottonwool-like nanocomposite (ECWN) is a novel synthetic bone substitute that incorporates amorphous calcium phosphate nanoparticles into a biodegradable synthetic copolymer poly(lactide-co-glycolide). The objectives of this study were to develop a tooth extraction socket model in sheep for bone graft research and to compare ECWN and bovine-derived xenograft (BX) in this model.

**Material and methods:** Sixteen crossbred female sheep were used. Bilateral mandibular premolars were extracted atraumatically. Second and third premolar sockets were filled (Latin-square allocation) with BX, ECWN or left unfilled. Resorbable collagen membranes were placed over BX and selected ECWN grafted sockets. Eight sheep per time period were sacrificed after eight & 16 weeks. Resin-embedded undemineralised sections were analysed for descriptive histology and histomorphometric analyses.

**Results:** At eight weeks, there were with no distinct differences in healing among the different sites. At 16 weeks, osseous healing followed a fine trabecular pattern in ECWN sites. Non-grafted sites showed thick trabeculae separated by large areas of fibrovascular connective tissue. In BX grafted sites, xenograft particles were surrounded by newly formed bone or fibrovascular connective tissue. There were no statistically significant differences in bone formation across the four groups. However, ECWN sites had significantly less residual graft material than BX sites at 16 weeks ( $p = 0.048$ ).

**Conclusions:** This first description of a tooth extraction socket model in sheep supports the utility of this model for bone graft research. The results of this study suggested that the novel material ECWN did not impede bone ingrowth into sockets and showed evidence of material resorption.

## Introduction

The alveolar process develops with the eruption of teeth and is dependent on distinctive individual anatomic and developmental factors (Schroeder 1986). Following tooth extraction, this bone resorbs, especially during the first six months (Lam 1960) leading to a saddle-shaped ridge in most cases. Schropp et al. (2003) identified a 50% post-extraction reduction in the width of the alveolar ridge, of which two-thirds occurred during the first three months. There is more loss in the bucco-lingual width than the corono-apical height of the alveolar ridge (Lekovic, et al. 1998). This can complicate the replacement of the missing tooth, since successful rehabilitation options need adequate bone height and width.

Alveolar ridge preservation is carried out to preserve the ridge volume within the bony envelope existing at the time of extraction (Hämmerle, et al. 2012). Various techniques have been proposed to carry out this procedure, which mostly involves the placement of a bone graft/substitute material into the extraction sockets.

Various animal and human studies have shown that some of these bone substitutes were able to limit but not eliminate the post-extraction alveolar ridge resorption to a certain extent (Ten Heggeler, et al. 2011). However, the quality of the new tissue formed within the socket may vary due to different healing patterns within the alveolar socket with different bone substitute materials. In this context, not only is the amount of the newly formed bone important in these grafted sites, but also the quality of osseous tissues in the socket area is essential, especially when the justification of ridge preservation is to facilitate the placement of a dental implant (Horváth, et al. 2013).

In the publications by Schneider and co-workers (2007, 2009 and 2011), the performance of a flexible, mouldable, electrospun cottonwool-like nanocomposite (ECWN) was assessed. This material incorporates amorphous calcium phosphate nanoparticles (a-CaP) into a biodegradable synthetic copolymer poly(lactide-co-glycolide) (PLGA). This material is prepared through an electrospinning process, which gives it the typical cotton wool-like appearance. This characteristic of the material allows easy proportioning, handling and adaption to a bone defect. The preclinical study showed high bioactivity of ECWN four weeks after implantation, with the formation of new bone and increased cell density. Resorption of the graft

material as early as 4 weeks after surgical placement was also reported in this same study. The authors highlighted the need for further investigations in animal models, to evaluate the long-term stability and clinical outcome of this material.

Therefore, the current study was designed

- 1) To establish and test a tooth extraction socket model for bone graft research in sheep and
- 2) To evaluate histomorphometrically the healing potential of ECWN in post-extraction tooth sockets, and
- 3) To compare this to the response to bovine xenograft as well as non-grafted spontaneous healing.

We claimed that a sheep model would prove suitable and useful for bone graft research. Our null hypothesis was that all materials would perform equally in terms of new bone formation and residual graft material determination.

## **Materials and Methods**

### **Animal selection and study design**

Ethical approval for this study was obtained from the Otago Animal Ethics Committee, protocol number AEC 65-11. Sixteen cross-bred ewes aged four to five years ( $76.7 \pm 6.5\text{kg}$ ) were used in this study. The animals exhibited an intact dentition with a healthy periodontium. Following selection, the sheep were treated to control parasites and immunized in preparation for surgery. The sheep were divided into two groups of eight with two healing periods, i.e. eight and 16 weeks, respectively. As no previous study had been conducted using this experimental model and material before, eight animals were included in each group in an effort to increase the statistical power of the study.

All sheep received the pre-operative antibiotic Trimethoprim (Amphoprim injection 1ml/15kg, Virbac New Zealand Ltd., East Tamaki, Auckland). General anaesthesia was induced by intravenous Thiopentone 20mg/kg (Bomac Laboratories Ltd., Manukau City, Auckland) and maintained by Halothane (1-2%) and Nitrous oxide/oxygen (1:2). The second and third mandibular premolars were selected as the

experimental sites, as the roots of these teeth resemble those of the human teeth. Only the premolars were selected because the surgical accessibility is limited past the third premolar without a cheek incision. The surgical sites were thoroughly cleansed using gauze soaked with 0.2% w/v chlorhexidine gluconate (Savacol<sup>®</sup>, Colgate-Palmolive, NZ).

### **Tooth extraction and grafting procedures**

The flap design involved intra-sulcular incisions from the mesial aspect of the first mandibular premolar to the distal aspect of the third premolar, on both the buccal and lingual aspects. Full thickness mucoperiosteal flaps were raised. A surgical piezotome unit (Piezosurgery<sup>®</sup>, Mectron, Genoa, Italy) with the extraction tip (EX1, EX2, EX3) was used to sever the buccal and lingual periodontal ligament of the premolars under copious irrigation with 0.9% sterile saline (Baxter Healthcare Pty Ltd., NSW Australia). The first to the third premolars were then elevated mesially using Coupland's and Cryer's elevators (Hu-Friedy, Henry Schein Shalfoon, Auckland, New Zealand). The teeth were extracted with considerable care in order not to fracture their roots or to damage the cortical plates. The extraction sockets were curetted and dental intra-oral radiographs were obtained using a long-cone paralleling technique.

Four treatments were provided to each animal, which were as follows: ECWN as a filler with a resorbable collagen membrane (OsseoGuard<sup>®</sup>, Biomet 3i, Palm Beach Gardens, USA, 15mm x 20mm; ECWN+), the ECWN without a membrane (ECWN-), a bovine xenograft (Endobon<sup>®</sup>, Biomet 3i) with a resorbable collagen membrane (B+; positive control) and non-grafted sites (No-; negative controls) as depicted in Figures. 1 and 2. Using Latin-square allocation, the second and third premolar extraction sockets of the 16 sheep were treated with one of four treatments. Each sheep received all four treatments at random, in a balanced way. All sockets were filled until the graft material reached the marginal bone crest. Primary closure was achieved at the extraction sites using a resorbable suture material (Vicryl 3-0, Ethicon Inc., Somerville, MA, USA). ECWN is a new prototype material. It is not registered and has therefore no CE mark yet. The test material was supplied by the Swiss Federal Institute of Technology Zurich (Functional Materials Laboratory, Institute for Chemical and Bioengineering, ETH Zurich, Switzerland) after receiving  $\gamma$ -radiation treatment.

Following surgery, all animals received anti-inflammatory Carprofen 5ml (Rimadyl® injection 50mg/ml, Zoetis, Mt Eden, New Zealand) and antibiotic medication (Trimethoprim 1ml/15kg) once daily for three days.

## **Euthanasia and histological processes**

After healing periods of 8 and 16 weeks, respectively, the animals were euthanized under general anaesthesia and perfused through the carotid arteries using 10% neutral buffered formalin (NBF; BioLab Ltd., Auckland, New Zealand). Following euthanasia and perfusion, the surgical sites were identified. The tissue specimens in the mandible were retrieved *en bloc* and the block specimens were photographed and placed into a sealed container of 10% NBF.

The individual extraction sockets were identified on the radiograph and matched on the mandible specimens. The sockets were separated using a manual coping saw (Spear and Jackson, England) and specimens were processed for non-demineralised embedding in methacrylate resin. All samples were processed according to the protocol described by Donath & Breuner (1982) and Duncan (2005). Briefly, specimens were dehydrated in ascending concentrations of alcohol, cleared in xylol and embedded in methylmethacrylate at room temperature. Embedded blocks were sectioned using an R330 diamond wheel on a Struers Accutom-50 precision cut-off saw (Intellection Pty Ltd, Australia) and glued to plastic slides using cyanoacrylate glue. For each specimen, two buccal-lingual sections representing the central area of the socket were selected. They were then further ground and polished using a rotating grinding machine (Tegra-Pol, Struers, Ballerup, Denmark) and Silicon Carbide Paper (grit size #180 to #4000) (Struers, Ballerup, Denmark). The final thickness of the sections was  $100\mu\text{m} \pm 10\mu\text{m}$ . After superficial etching and decalcification with 20% ethanol and 1% formic acid in an ultrasonic bath, all sections were surface stained with one part MacNeal's tetrachrome (methylene blue, azur II and methyl violet) and two parts toluidine blue.

## **Descriptive histology and histomorphometric analyses**

Images were obtained and digitalised using a light microscope (Olympus AX70, Olympus Optical Co. Ltd, Japan) and an imaging system (Micropublisher 5.0 RTV, Qimaging) at a 10-fold magnification. A region of interest (ROI) measuring 4 x 6 mm

was identified for each specimen using the following protocol (Fig. 3): The coronal margin of the area was aligned with the level of the alveolar crest. An effort was made to avoid the alveolar bone proper and the cortical bone to be included in the ROI. These regions were chosen because they represent the portion of the alveolar ridge most likely to be utilized for implant placement.

For each specimen, the area occupied by newly formed bone (NB), residual bone graft material (RG), and fibrovascular connective tissue (FCT) were measured using full-color thresholding on a computer-based image analysis system, Image J (version 1.47q, NIH, Chicago, USA). A semi-automated segmentation technique was used to calculate the different tissue volumes. Threshold values for NB, RG and FCT were selected manually according to the signal intensity in each image. These segmented tissues were measured and expressed as area percentages within the ROI. Measurements were taken from the two buccal-lingual sections representing the central area of each socket. The mean value of these two sections was used to calculate the overall mean results for each treatment type.

Intra-examiner reliability for the morphometric evaluation was assessed using the two-way mixed model for intra class correlation coefficient, with the software package SPSS statistics software for Mac (version 20.0, IBM corporation, Somers, USA). Duplicate measurements at two weeks apart were completed using eight randomly selected samples of the specimens. The concordance correlation coefficient for the morphometric measurements ranged from 0.87 to 0.98. The two sets of data are highly correlated to each other. There were no statistically significant differences between each pairs of measurements.

## **Statistical analysis**

Mean values, standard deviations and medians were calculated for all variables using the sheep as a statistical unit. The differences between the test and control groups were analyzed by using a mixed model analysis with repeated measures for the treatment factor, and a compound symmetry covariance matrix. This model of analysis takes into account both the fixed effect (treatment groups and healing time) and the random effect (between the experimental animals) and allows for missing values in the dataset. Since some apparent variability among treatments was noted, compound symmetry heterogeneous and unstructured covariance forms were



investigated. But this was rejected on the grounds of the increased number of parameters used (7 and 9 respectively) with a maximum of 23 data points available for the 16 week analysis. Normality of residuals from all fitted models was checked and found to be satisfactory.

Differences were considered statistically significant when  $p$  was  $<0.05$ . Pairwise comparisons with Bonferroni adjustment were conducted for the groups with statistically significant differences. The statistical analysis was performed with SPSS statistics software for Mac (version 20.0, IBM corporation, Somers, USA).

## **Results**

### **Descriptive histology**

Four extraction socket sites were harvested from each experimental animal. A total of 64 sites were processed for resin embedding. Two extraction sockets from a left hemi-mandible of one animal did not heal. Bone sequestrum was detected within the sockets, disrupting the complete epithelialisation over the bony defects. Retained root tips were detected in four extraction sockets. Therefore, six of the specimens were not included in the evaluation: five from the eight weeks group and one from the 16-week group.

#### **Eight week group**

Extraction socket healing at the sites grafted with ECWN and resorbable membrane (ECWN+) was composed of predominantly woven bone (Fig. 4a and 5a). The orientation of collagen fibres within the newly formed bone was irregular. The fibrovascular connective tissue was immature and vascular with some inflammatory cell infiltrate. The residual ECWN graft material was intimately associated with the newly formed bone. Some of the ECWN graft material was incorporated into the osteoid.

Extraction sockets grafted with ECWN alone healed in a similar pattern to ECWN+ sites. Irregular trabecular bone was formed in the defects, mostly of woven bone (Fig. 4b and 5b). In contrast to that found in ECWN+ sites, the margin of the newly formed

bone did not extend above the buccal and lingual bone crests. Residual graft material was observed in close proximity with newly formed bone.

In the specimens grafted with BX, most of the graft particles were found in the coronal portion of the extraction sockets (Fig. 4c and 5c). BX particles were surrounded by woven bone and connective tissue. There was no bone tissue bridging detected across the coronal margin of the defect in most of the specimens.

In the non-grafted sites, islands of loose trabecular bone were seen in the extraction sockets. Immature woven bone was also seen across the coronal margin of the defect, connecting the buccal and lingual cortical plates (Fig. 4d).

### **Sixteen week group**

Healing within the extraction sockets continued to mature by 16 weeks. At the ECWN+ and ECWN- sites, finger-like bone trabeculae were observed within the grafted sites. The newly-formed bone sealed the extraction socket entrance and bridged across the buccal and lingual cortical plates in all samples (Fig. 6a and 6b). Woven bone was replaced by lamellar bone with parallel collagen fibres. The fibrous connective tissue stroma became less vascular with less inflammatory cells. ECWN graft material was almost undetectable (Fig. 7a and 7b).

Healing within BX grafted extraction sockets appeared to be different at 16 weeks, when compared to ECWN grafted sites. New bone could be detected throughout the different levels of the extraction sockets (Fig. 6c and 7c). A hard tissue bridge formed at the coronal margin of four out of the eight extraction sockets contained BX graft particles. In the other half of the specimens, no hard tissue bridge could be detected. The coronal margins of the entry to these extraction defects were occupied by BX particles surrounded by connective tissue matrix. Most of the particles were surrounded by either a mixture of woven/lamellar bone or connective tissue. Some of the BX particles, especially in the coronal and apical portion of the defects, were surrounded by connective tissue only. Newly formed bone was predominantly found adjacent to the graft particles.

At 16 weeks, healing at non-grafted sites was characterised by the formation of thick bony trabeculae extending from the buccal and lingual cortical plates into the bony defects (Fig. 6d). These trabeculae were separated by larger fibrous stroma space

when compared to ECWN grafted sites. The newly formed bone had parallel collagen fibres, resembling that of lamellar bone. This healing pattern was distinctly different from that found in ECWN and BX grafted sites.

## **Histomorphometric analysis**

### **Eight week group**

Results for each type of treatment after eight weeks of healing are presented in Table 1. The amount of newly formed bone in ECWN grafted sites was  $36.5 \pm 17.3\%$  for ECWN+ sites and  $40.7 \pm 16.9\%$  for ECWN- sites. The percentage of new bone detected in the eight weeks extraction sockets was highest in the NO- sites ( $47.9 \pm 5.0\%$ ). The amounts of newly formed bone in the grafted sockets were similar, with BX sites showing the greatest variation ( $38.6 \pm 18.1\%$ ). The statistical comparison regarding the four different treatment modalities did not reveal any significant differences.

Comparison of the residual graft materials found in the different sites also revealed no significant differences. Little residual graft material ( $<5\%$ ) was detected in all grafted sites.

### **16 week group**

Results for each type of treatment after 16 weeks of healing are presented in Table 2. New bone formation within the ECWN- sites ( $45.2 \pm 6.8\%$ ) was again similar to NO-sites ( $45.7 \pm 10.7\%$ ). However, ECWN+ sites revealed the highest percentage of newly formed bone ( $49.8 \pm 12.5\%$ ), whereas BX sites showed the lowest amount of new bone ( $41.1 \pm 11.1\%$ ).

The only borderline statistically significant difference was found for the amount of residual graft between ECWN grafted sites without membrane and BX sites ( $p = 0.048$ ). Residual graft materials detected within BX sites also showed greater variation between the experimental animals ( $5.4 \pm 5.6\%$ ).

## Discussion

Although there is a growing literature supporting the use of sheep or goats for research into oral implants and associated bone replacement grafting procedures (Potes et al. 2008), it must also be noted that sheep bone differs from canine and human bone as it is denser and stronger (Nafei et al. 2000) and contains a higher proportion of primary osteons (Eitel et al. 1981, deKleer 2006). Although sheep animal models have been presented for mandibular critical size defect healing (Salmon & Duncan 1997), dental implant research (Duncan 2005, Vlamminck, et al. 2008, Campbell & Duncan 2013), and in studies of periodontitis and periodontal defects (Duncan, et al. 2003, Baharuddin 2010), differences between the response in the animal model and that found in human subjects were noted in these publications. Others have reported no appreciable difference in the healing of tibial or femoral orthopaedic devices between dogs and sheep (Lippuner et al. (1992). Reviews by Martini et al (2001) and Pearce et al. (2007) summarising works by a number of authors, concluded that the sheep is a valuable model for human osseous healing and remodelling. An and Friedman (1999) commented that “for most orthopaedic animal studies, there is no specific reason for dogs to be used when goats and sheep are also available”.

It has been suggested that anatomical location has an influence on the degree of similarity or otherwise for osseous healing when sheep are compared with humans (Pearce et al. 2007). Fluorescence studies of mineral apposition rates around dental implants installed into the posterior mandible of sheep demonstrated rates approximately 1.3 times greater than comparable published results for human mandible (Duncan, 2005). The healing time points chosen for the current study were eight and 16 weeks, which corresponds to 11 and 21 weeks of wound healing in humans. The chosen time periods in our study reflected the extraction socket healing at the time of implant placement in humans, since dental implants are usually placed into grafted extraction sockets after three to seven months of healing (De Coster, et al. 2011, Barone, et al. 2012, Gholami, et al. 2012).

Although to our knowledge, the current study is the first published account of bone replacement grafting using the sheep mandibular tooth socket, others have published

work after grafting into defects created in the sheep mandible (Gatti et al. 1990, Gatti & Zaffe 1991a&b) and in the sheep maxillary sinus, with or without dental implants (Haas et al. 1998, 2002a&b, Jaske 2003, Phillip et al. 2013). Experimental work from our institution has demonstrated similarities in the healing of furcation defects created adjacent to sheep mandibular premolar teeth (Pack, 1997; Danesh-Meyer et al., 1995; Whelan et al. 1997; Mohammed et al. 1998; Baharuddin et al. 2014); various sheep animal models for periodontal and peri-implant healing have been recently reviewed (Duncan, 2014). We believe that this new preclinical model utilising extraction sockets in sheep yields valuable information with respect to osseous healing around bone replacement grafts, but we acknowledge that due considerations of differences between this animal species and our human patients must be borne in mind when interpreting the results.

This was the first extraction socket healing study in sheep. Tooth extractions in sheep are known to be extremely challenging in sheep (Duncan 2005, Vlaminck et al. 2008). Therefore, full thickness periosteal flaps were elevated. This was necessary to ensure adequate access and better visibility of the teeth to prevent root fracture. However, we agree that the use of full thickness flaps in periodontal surgery has been reported to increase the alveolar crestal bone loss (Wood et al. 1970, Staffileno 1974). Fickl and colleagues (2008b) have shown the association between full periosteal elevation and increased buccal lingual alveolar resorption following tooth extraction in a dog study.

The grafting procedure carried out in the present study was similar to the guided tissue regeneration procedures. Primary wound closure was achieved to enclose the graft material and collagen membrane. There were abundant gingival tissues in sheep to achieve primary closure without the need of coronal advancement of the periosteal flaps. Another reason to raise periosteal flaps was to prevent infection of the collagen membranes as we are dealing with ruminant animals.

In this study, a semi-automated segmentation technique was used to calculate the different tissue volumes. Threshold values for NB, RG and FCT were selected manually according to the signal intensity in each image. These segmented tissues were measured and expressed as area percentages within the ROI. The semi-automated segmentation technique has been compared with the classical point-counting stereology in its application in histomorphometric analysis and immunohistochemical cell counting (Amenábar et al., 2006; Montgomery et al.,

2008). The authors compared the intra-reader and inter-reader variability and reported no statistically significant differences between the two methods. In the current study, we used the slides taken from the central part of the socket. In doing so, we accepted the assumption that healing in the central part of the socket is representative of the whole extraction socket healing.

Extraction socket healing has been studied extensively in animal (other than sheep) and human studies (Amler 1969, Cardaropoli et al. 2003). The healing events of the current study are consistent with those described by Cardaropoli et al. (2003) in a study carried out in the dog model. They reported that healing at 60 days (around eight weeks) was characterised by the formation of a hard tissue bridge separating the marginal mucosa from the extraction socket and composed mainly of woven bone. After 120 days (around 16 weeks), the marginal hard tissue bridge was reinforced by layers of lamellar bone. It is therefore likely that the healing times in sheep and dog are similar, as reported by Duncan (2005).

The pattern of healing within the ECWN grafted sites was distinctly different from that found in non-grafted sites. Fine finger-like bone trabeculae were observed within the grafted sites. The striae of bone trabeculae were close to each other and extended from the buccal and lingual cortical plates towards the middle of the extraction sockets. Therefore, our findings are in agreement with previous research reporting the histology of osseous healing following grafting of this material (Schneider et al. 2009, 2011). In these studies, ECWN was grafted into rabbit calvarial and sheep femur defects. After 4 and 8 weeks of healing, the authors also reported that the newly-formed bone within the ECWN grafted defects showed a finer, more spongy appearance. The authors also reported that small round pores were observed in the newly formed bone tissue in the ECWN grafted defects. A possible explanation for this distinct healing pattern might be due to the osteoconductive property of ECWN. A previous *in vitro* study (Schneider et al. 2007) demonstrated that the ECWN scaffold supported the formation of a continuous hydroxyapatite (HAp) layer onto the graft material. As HAp particles were deposited onto the graft material, the mineralization process led to the formation of fine striae of trabeculae within the grafted extraction sockets or bone defects.

In contrast, the osteoconductive properties of BX have been reported in various animal and human studies (Fugazzotto 2003, Araújo & Lindhe 2009, Mardas, et al.

2010). A common finding from previous research was that the material was not resorbed or eliminated from the grafted sites (Artzi, et al. 2000, Molly, et al. 2008, Smith 2011). The present histological evaluation of the 16 weeks specimens is corroborated by the findings reported in other animal studies (Araújo, et al. 2008, Fickl, et al. 2008a). These authors reported that most of the graft particles were in direct contact with woven and lamellar bone after three to four months of healing. A small part of the biomaterial was also surrounded by connective tissue, especially at the most coronal part of the extraction defect.

ECWN+ sites revealed the highest percentage of new bone ( $49.8 \pm 12.5\%$ ) amongst the four treatment modalities after 16 weeks of healing. As this study was the first animal study to investigate ECWN material in extraction socket healing, no comparison with previous histology was possible. In the current study, the differences reported in the percentage of new bone formation within the grafted and non-grafted sites were not found to be statistically significant. The studies carried out by Schneider et al. (2009 and 2011) also did not find any statistical differences in the histophotometric analysis. The lack of statistical significant differences in our study may be due to the small test group sizes and the large variations for the percentage of newly formed bone obtained from the 16 sheep. These variations may be sheep dependant and caused by the different healing capacities between individual animals. The considerable variation in bone formation within the sockets evaluated might be due to a difference in individual factors influencing bone physiology.

The amount of residual BX graft material was very low at eight weeks in the current study, measuring only  $2.5 \pm 0.9\%$ . This result differed from other published animal studies. After three months of healing, Araújo et al. (Araújo & Lindhe 2009, Araújo & Lindhe 2011) found the residual BX graft material occupied  $12.2 \pm 9.1\%$  to  $24.6 \pm 3.7\%$  of the measured regions of interest. These studies were conducted using the same animal model at different times. A possible explanation for the discrepancy between our result and those reported in the literature may be due to a quite profuse bleeding from the extraction sockets during surgery. This may have led to the premature loss or migration of graft particles. Some authors have already speculated that the ensuing blood flow into the cone-shaped extraction socket may have forced BX graft particles towards the coronal direction (Araújo & Lindhe 2009). Therefore,

the apical portion of the extraction socket was void of graft particles and healed in the same way as the non-grafted sites.

However, at 16 weeks significantly more residual graft materials were still observed in BX sites than ECWN grafted sites with or without membrane ( $p = 0.055$  and  $p = 0.048$  respectively). This may be explained by the different resorption rates and mechanisms of the two graft materials. The degradation of ECWN and ECWN-like material has been tested *in vitro* (Loher, et al. 2006, Schneider, et al. 2007). After the material was placed into simulated body fluid for two weeks, a continuous HAp layer with a thickness of about  $2\mu\text{m}$  was already detected. Two processes were reported to be occurring simultaneously, PLGA degradation through polymer hydrolysis and the deposition of hydroxyapatite on the surface of the fibres. Although no study has been conducted to define the time taken for ECWN to be resorbed and replaced, previous *in vivo* studies reported partial degradation after four weeks of healing in rabbit calvarial defects and murine chest wall replacement (Schneider, et al. 2009, Jungraithmayr, et al. 2014)). ECWN degradation was reported to be faster when implanted as a chest wall replacement in the pleural cavity than in subcutaneous tissues. Another degradation and bioactivity study was carried out by the same group on a similar co-polymer prepared by solvent casting (Loher, et al. 2006). They reported the degradation of around 7% of the composite mass after six weeks immersion in simulated body fluid. On the other hand, the complete resorption of BX particles has been questioned in the literature. Some studies have reported the partial or complete resorption of BX particles (Thaller, et al. 1994, Fugazzotto 2003), while others showed negligible resorption (Artzi, et al. 2000, Smith 2011). BX particles were still detectable after 11 years in a human maxillary sinus study (Mordenfeld, et al. 2010). The incorporation of BX particles in extraction socket healing involves a series of processes (Araújo, et al. 2010). The biomaterial is initially exposed to surface resorption by osteoclasts. It has been proposed that osteoclasts found adjacent to BX particles function like macrophages to clean and prepare the graft surface for the deposition of new bone (Jensen, et al. 2006). However, the biomaterial itself is not engaged in the processes of resorption or degradation, which may explain the higher proportion of residual graft in BX sites compared to ECWN sites.

This first description of a tooth socket model in sheep supports the overall utility of this model for bone graft research. The novel bone substitute material ECWN



combined the flexibility of PLGA polymer fibres with the bioactivity of calcium phosphate ceramic. As the demand for flexible and easy-to-apply implant material for the repair of complex-shaped bone defects is increasing, the current study will serve as a base for applying this novel material in future studies.

Other properties determine the success of a bone graft/substitute material include material degradation and new bone formation. The current study also demonstrated the biocompatibility and resorption of ECWN. Its osteoconductive property was revealed through the pattern of histological healing within the extraction sockets. The *in vivo* performance of the material did not impede bone formation within the extraction defects. These findings add substantially to our understanding of this material and warrant further investigations of ECWN in bony defects in periodontal, implant and peri-implant research.

## **Conclusion**

This first description of a tooth socket model in sheep supports the utility of this model for bone graft research. The results of this study suggested that the novel material ECWN did not impede bone ingrowth into sockets and showed evidence of material resorption. The present study also confirmed previous findings where new bone was formed to encapsulate BX particles.

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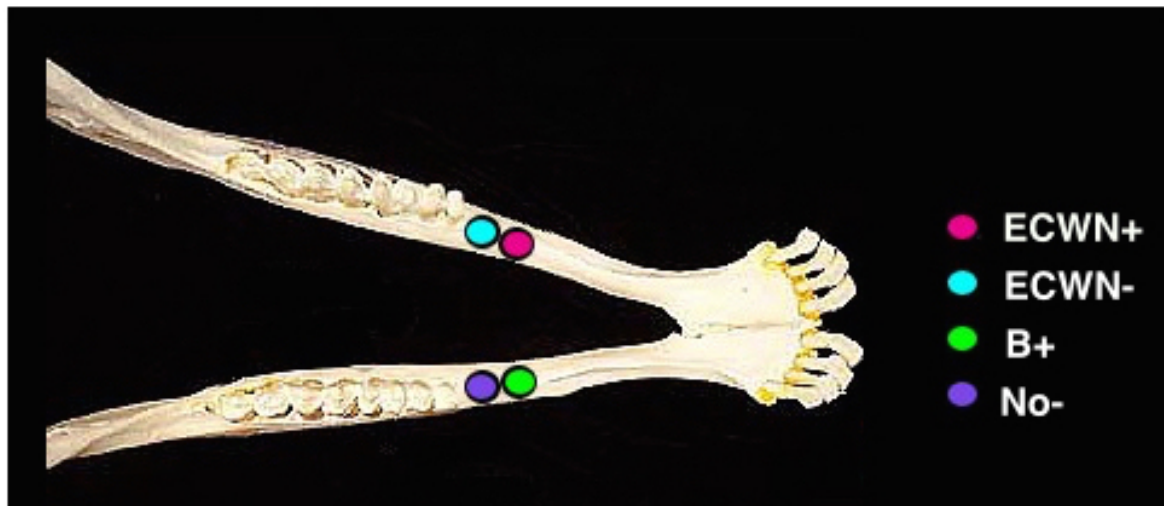
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**Table 1 Planimetric data describing the composition (%) of tissues in the grafted and non-grafted extraction sites at eight weeks**

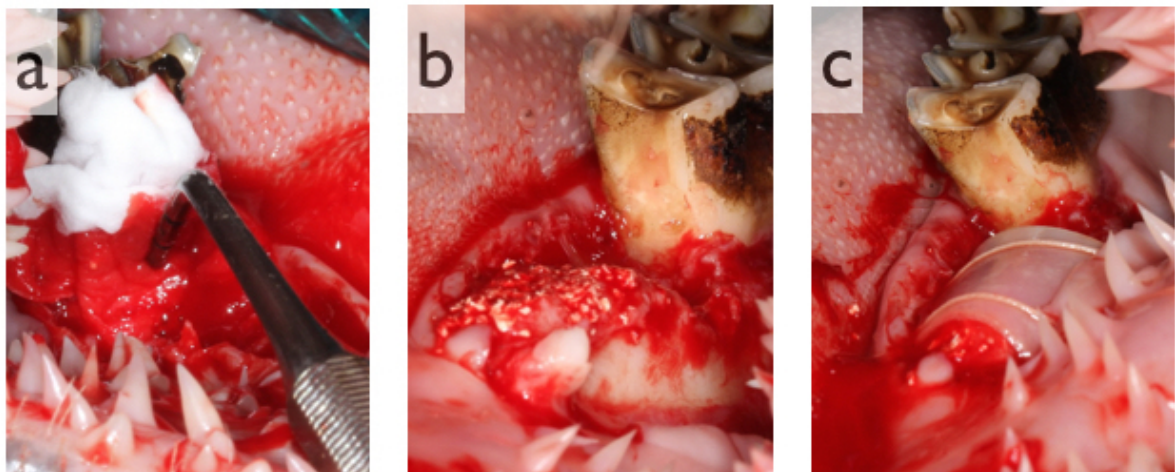
Treatment type	No. of specimens	Newly formed bone (%) Mean $\pm$ SD Median (95%CI)	Residual graft (%) Mean $\pm$ SD Median (95%CI)	Fibrovascular connective tissue (%) Mean $\pm$ SD Median (95%CI)
ECWN+	6	40.7 $\pm$ 16.9 45.6 (23.0-58.5)	3.0 $\pm$ 1.8 2.8 (1.1-4.8)	57.7 $\pm$ 16.2 53.5 (40.6-74.7)
ECWN-	5	36.5 $\pm$ 17.3 32.5 (15.0-57.9)	1.6 $\pm$ 0.8 1.5 (0.6-2.5)	61.7 $\pm$ 18.0 65.5 (39.3-84.1)
B+	8	38.6 $\pm$ 18.1 43.6 (23.5-53.8)	2.5 $\pm$ 0.9 2.2 (1.8-3.3)	59.7 $\pm$ 18.0 54.9 (44.7-74.8)
NO-	8	47.9 $\pm$ 5.0 49.0 (43.7-52.0)		52.9 $\pm$ 4.7 51.9 (49.0-56.9)

**Table 2 Planimetric data describing the composition of tissues in the grafted and non-grafted extraction sites at 16 weeks**

Treatment type	No. of specimens	Newly formed bone (%) Mean $\pm$ SD Median (95%CI)	Residual graft (%) Mean $\pm$ SD Median (95%CI)	Fibrovascular connective tissue (%) Mean $\pm$ SD Median (95%CI)
ECWN+	8	49.8 $\pm$ 12.5 46.5 (39.4-60.3)	1.2 $\pm$ 0.7 1.3 (0.6-1.8)	49.0 $\pm$ 12.1 52.1 (38.9-59.1)
ECWN-	8	45.2 $\pm$ 6.8 46.5 (39.6-50.9)	1.1 $\pm$ 0.7* 1.2 (0.5-1.7)	53.7 $\pm$ 6.4 53.2 (48.3-59.1)
B+	7	41.1 $\pm$ 11.1 39.4 (30.8-51.4)	5.4 $\pm$ 5.6 3.5 (0.2-10.61)	53.5 $\pm$ 10.4 54.5 (43.9-63.2)
NO-	8	45.7 $\pm$ 10.7 44.0 (36.7-54.6)		54.3 $\pm$ 10.7 56.0 (45.4-63.3)

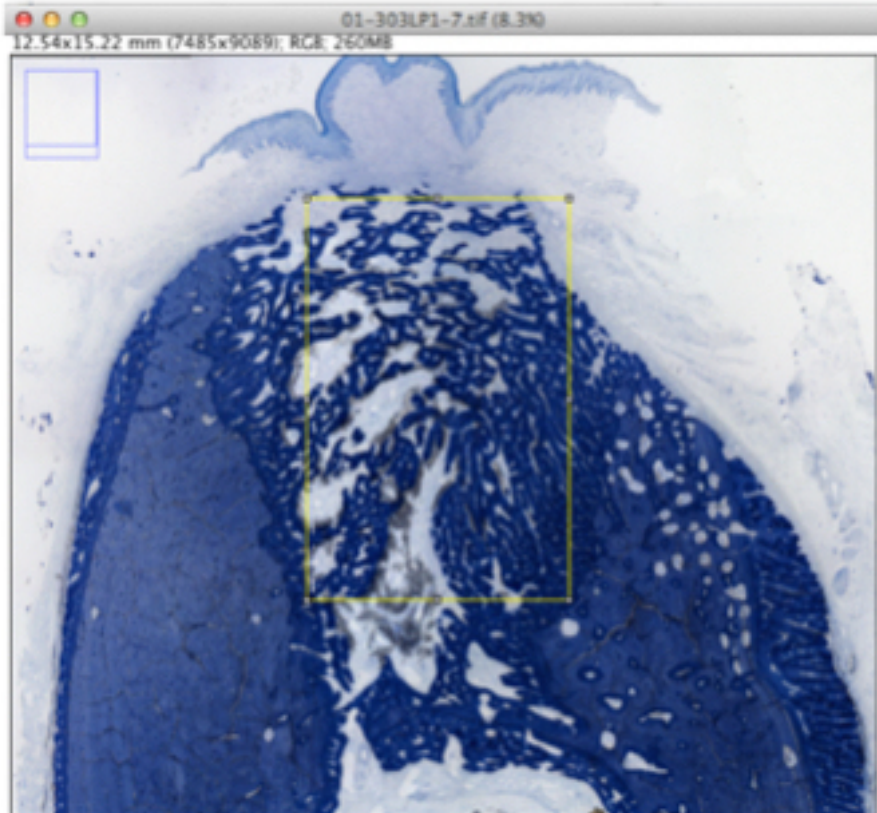


*Fig. 1.* A schematic drawing of the four treatment groups

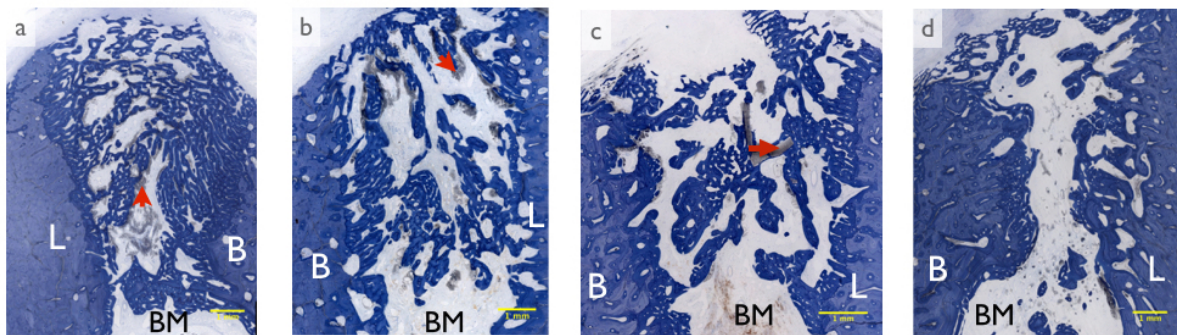


*Fig. 2.* Clinical photographs illustrating the socket sites treated with: (a) ECWN; (b) BX; (c) resorbable collagen membrane.





*Fig. 3.* Selected region of interest (ROI) measured 4x6 mm



*Fig. 4.* Resin-embedded histology for the buccal-lingual sections of the extraction sockets, at eight weeks (MacNeal's tetrachrome and Toluidine blue stain, 10x magnification: (a) ECWN+ site; (b) ECWN- site; (c) B+ site; (d) NO- site. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space; Red arrows, residual graft. Scale bar = 1mm



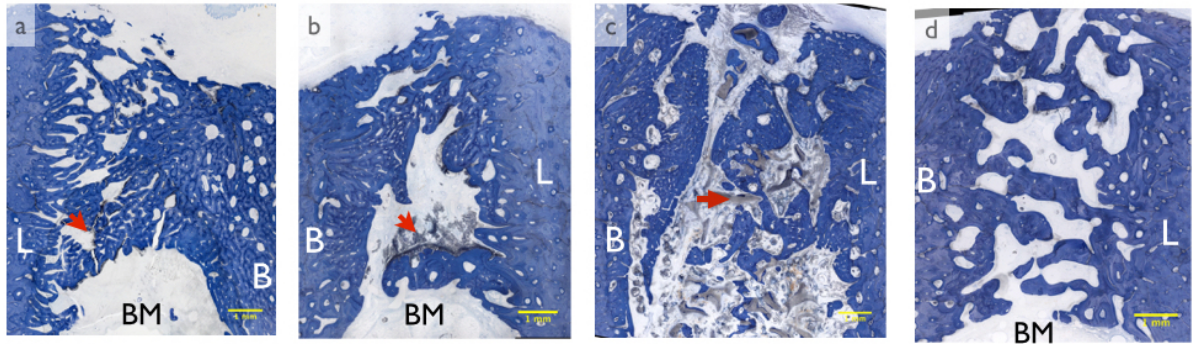


Fig. 5. Resin-embedded histology for the buccal-lingual sections of the extraction sockets, at 16 weeks (MacNeal's tetrachrome and Toluidine blue stain, 10x magnification): (a) ECWN+ site; (b) ECWN- site; (c) B+ site; (d) NO- site. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space; Red arrows, residual graft. Scale bar = 1mm

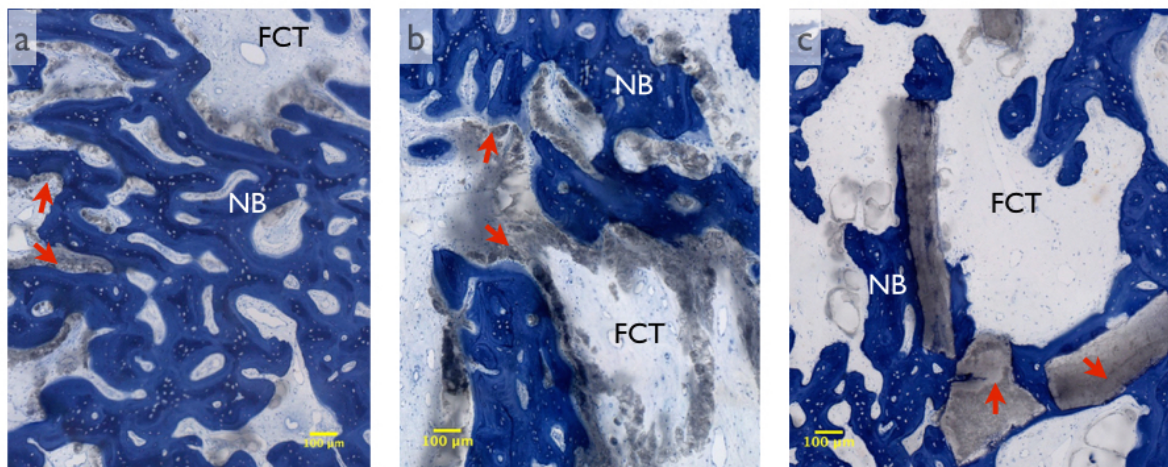


Fig. 6. Resin-embedded histology of the test groups, at eight weeks (MacNeal's tetrachrome and Toluidine blue stain, 100x magnification): (a) ECWN+ site; (b) ECWN- site; (c) B+ site. NB, Newly formed bone; FCT, Fibrovascular connective tissue; Red arrows, residual graft. Scale bar = 100µm

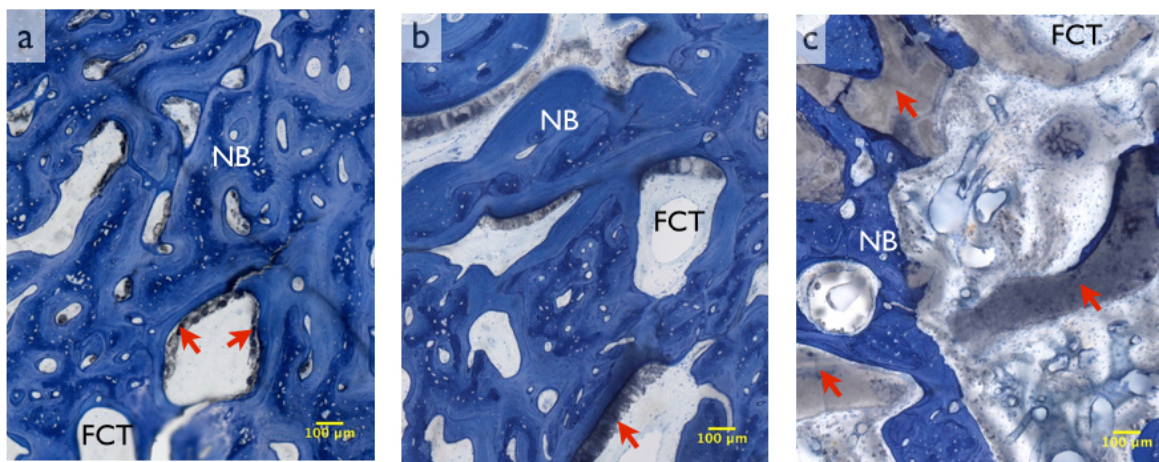


Fig. 7. Resin-embedded histology of the test groups, at 16 weeks (MacNeal's tetrachrome and Toluidine blue stain, 100x magnification): (a) ECWN+ site; (b) ECWN- site; (c) B+ site. NB, Newly formed bone; FCT, Fibrovascular connective tissue; Red arrows, residual graft. Scale bar = 100µm